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(54) METHOD OF TRANSFORMING MONOCOTYLEDON.

(57) A method of transforming monocotyledon which necessitates only a short period from the transformation to the generation of a plant body as compared with the conventional methods, thus reducing the frequency of occurence of mutants, and can be generally applied to the plant for which any system of regenerating the plant body from the protoplast has not been established, and in which the material to be used can be readily prepared. The method comprises transforming cultured tissues of a monocotyledon under or after dedifferentiation with a bacterium of the genus *Agrobacterium* containing desired genes.

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1988: Bio/Technol. 6:185-189), and that virC gene in the plasmid of Agrobacterium is indispensable to the infection (Grimsley et al., Mol. Gen. Genet. 217:309-316).

Gould J. et al. (1991; Plant Physiol. 95:426-434) inoculated super-virulent Agrobacterium EHA1 having a kanamycin-resistant gene and a GUS gene to shoot apices of maize after injuring the shoot apices with a needle, and selected the shoot apex based on the resistance to kanamycin. As a result, plants having resistance to kanamycin were obtained. They confirmed by Southern blotting analysis that some of the seeds of the subsequent generation of the selected plants had the introduced gene (chimera phenomenon).

Mooney P.A. et al., (1991; Plant Cell, Tissue, Organ Culture 25:209-218) tried to introduce kanamycin-resistant gene into embryos of wheat using Agrobacterium. The embryos were treated with an enzyme to injure the cell walls, and then Agrobacterium was inoculated. Among the treated calli, although very small number of calli which were assumed to be transformants grew, plants could not be regenerated from these calli. The existence of the kanamycin-resistant gene was checked by Southern blotting analysis. As a result, in all of the resistant calli, change in structure of the introduced gene was observed.

Raineri et al. (1990; Bio/Technol. 8:33-38) inoculated super-virulent Agrobacterium A281 (pTiBo542) to 8 varieties of rice after injuring the scutella of the rice plants. As a result, growth of tumor-like tissues was observed in two varieties, Nipponbare and Fujisaka 5. Further, an Agrobacterium containing a plasmid having a T-DNA from which a hormone-synthesizing gene was removed and instead, a kanamycin-resistant gene and GUS gene were inserted therein was inoculated to embryos of rice. As a result, growth of kanamycin-resistant calli was observed. Although the expression of GUS gene was observed in these resistant calli, transformed plants could not be obtained from the calli. They interpreted these results as that the T-DNA was introduced into rice cells.

Thus, although the experimental results which suggest that introduction of genes into the plants belonging to family Gramineae such as rice, maize and wheat can be attained by using Agrobacterium have been reported, fully convincing results have not been obtained about the reproducibility, introduction efficiency and about the confirmation of the introduction of the gene (Potrykus I. 1990; Bio/Technol. 8:535-543).

As mentioned above, introduction of genes into the plants belonging to family Gramineae is now mainly carried out by the electroporation method. However, with this method, since protoplasts are used, a long time and much labor are required to obtain regenerated plants. Further, there is a danger that mutants may emerge at a high frequency due to the long culturing period. Still further, this method cannot be applied to the plants such as maize for which the system for regenerating plants from protoplasts has not been established. In view of this, as mentioned above, as for maize, it has been tried to use the apical meristem. However, the operation for isolating the apical meristem requires much labor and it is not easy to prepare apical meristem in a large amount.

Disclosure of the Invention

Accordingly, an objective of the present invention is to provide a method for transforming monocotyledons, with which the time required for obtaining regenerated plants from the time of transformation is shorter than that in the conventional methods, which can be generally applied even to the plants for which the systems for regenerating plants from protoplasts have not yet been established, and with which the preparation of the materials to be used is easy.

The present inventors intensively studied the influences of the monocotyledonous plant tissues treated with Agrobacterium, treatment conditions of Agrobacterium, constitution of the binary vector and the like on the introduction efficiency of the genes to discover that cultured tissues of monocotyledons can be transformed by using Agrobacterium with drastically high efficiency and reproducibility and that by employing this method, the above-mentioned object can be attained, thereby completing the present invention.

That is, the present invention provides a method for transforming a monocotyledon comprising transforming a cultured tissue during dedifferentiation process or a dedifferentiated cultured tissue of said monocotyledon with a bacterium belonging to genus Agrobacterium containing a desired gene.

By the present invention, it was first attained to introduce foreign genes to monocotyledons such as plants belonging to family Gramineae including rice, maize, wheat, barley and the like with good reproducibility. Although methods for transforming monocotyledons using Agrobacterium are known, they are not established methods as mentioned above. In contrast, according to the present invention, by inoculating Agrobacterium to cultured tissues which have not been employed in the conventional methods, genes can be very easily introduced. In the present invention, since a cultured tissue such as a callus which can be easily prepared is used, the sample materials can be obtained more easily than the conventional method

cloned. In pTOK154, between two border sequences of the T region, a kanamycin-resistant gene is inserted as a gene to be introduced into a monocotyledon. This is an example wherein the gene desired to be introduced into the monocotyledon is arranged in a plasmid having the cloned DNA fragment originated from the virulence region of pTiBo542. In Fig. 1, each reference symbol represents the following meaning:

SP: spectinomycin-resistant gene
HPT: hygromycin-resistant gene
NPT: kanamycin-resistant gene
TC: tetracycline-resistant gene
IG: intron GUS gene
BR: right border sequence of T-DNA
BL: left border sequence of T-DNA
virB, virC, virG: vir regions originated from super-virulent Agrobacterium tumefaciens A281
ORI: replication origin of ColE1
COS: COS site of λ phage
K: restriction enzyme Kpn I site
H: restriction enzyme Hind III site

The gene which is desired to be incorporated into the monocotyledon may be inserted into a restriction site in the T region of the above-described plasmid, and the desired recombinant plasmid may be selected depending on an appropriate selection marker such as drug resistance and the like which the plasmid has. However, if the vector, like pTOK162 shown in Fig. 1, is large and has a number of restriction sites, it is not always easy to insert the desired DNA in the T region of the vector. In such a case, the desired DNA can be inserted in the T region of pTOK162 by utilizing the in vivo homologous recombination (Herrera-Esterella L. et al, 1983; EMBO J. 2:987-995, Horch R. H. et al. Science 1984; 223:496-498) in the cells of Agrobacterium tumefaciens. That is, pTOK162 is preliminarily introduced into Agrobacterium tumefaciens and the plasmid pBR322 (or a similar plasmid) containing the desired gene is further introduced into Agrobacterium tumefaciens. Since pTOK162 has a region homologous with a region of pBR322, the pBR322 derivative containing the desired gene is inserted into pTOK162 by the genetic recombination via the homologous regions. Unlike pTOK162, pBR322 cannot replicate by itself in Agrobacterium tumefaciens. Therefore, pBR322 can only be alive in Agrobacterium tumefaciens in the inserted form in pTOK162 (the recombined pTOK162 and pBR322 is hereinafter designated "pTOK162::pBR322 derivative"). By selecting the transformants based on the selection markers (such as drug resistance) specific to each of pTOK162 and pBR322 derivative, Agrobacterium tumefaciens transformants containing pTOK162::pBR322 derivative may be obtained. The present inventors made a study by introducing various plasmids into Agrobacterium tumefaciens containing pTOK162, to discover that as the selection marker of the pBR322 derivative, spectinomycin-resistant gene (SP) originated from transposon Tn7 (De Greve H.H. et al., 1981; Plasmid 6:235-248) is suited. Thus, in cases where the desired gene has already been cloned into pBR322, by inserting SP gene into the plasmid, the desired DNA can be inserted in the T region of pTOK162 by homologous recombination in vivo in Agrobacterium tumefaciens. Alternatively, a plasmid containing the DNA from pBR322 and SP gene is first provided, and the desired gene may be inserted into this plasmid. In this case, by utilizing the border sequences of the T region, it is possible to arrange the kanamycin-resistant gene and the desired gene in separate T regions in pTOK162. When plants are transformed using the resistance to kanamycin as a marker, there is a substantial probability that both T regions are introduced, the introduction of the desired gene can be sufficiently attained. Further, in this case, since both T regions may be inserted into different chromosomes, it may be possible to subsequently segregate the desired gene from the kanamycin-resistant gene.

The gene which is desired to be introduced to monocotyledons is not restricted at all and may be any gene which can give a desired character. Examples of the desired gene include herbicide-resistant genes, antibiotic-resistant genes, virus coat protein genes for giving resistance to the virus, genes related to starch formation in albumen and the like, although the desired genes are not restricted thereto.

As the host bacterium belonging to genus Agrobacterium, Agrobacterium tumefaciens may preferably be employed, although not restricted.

Introduction of a plasmid into the bacterium belonging to genus Agrobacterium such as Agrobacterium tumefaciens can be carried out by a conventional method such as triple cross method of bacteria (Ditta G. et al., 1980; Proc. Natl. Acad. Sci. USA 77:7347-7351).

Since the Agrobacterium prepared as mentioned above has a highly virulent DNA originated from pTOK162, transformation of monocotyledons can be attained with a high efficiency.

It should be noted that in the method of the present invention, although the gene which is desired to be introduced into the monocotyledon is arranged between border sequences of the T region as in the prior

salts (Murashige and Skoog 1962; Physiol. Plant. 15:473-497), 0.5 g/l of casamino acid, 1 mg/l of 2,4-D, 0.2 mg/l of kinetin, 0.1 mg/l of gibberellin and 20 g/l of sucrose) and the cells were cultured therein at 25°C in the dark under shaking of 120 rpm to obtain suspended cultured cells. The medium was replaced with fresh medium every week.

(2) Ti Plasmid (Binary Vector)

Hygromycin resistant gene (HPT) and β-D-glucuronidase (GUS) gene were inserted in the T-DNA region of Ti plasmid to obtain the following plasmids:

(i) pIG121 Hm:

A plasmid in which the GUS gene containing the first intron of the catalase gene of caster bean and a hygromycin-resistant gene were ligated (Nakamura et al., 1991; Plant Biotechnology II (Extra Issue of GENDAI KAGAKU, pp.123-132), presented from Dr. Nakamura of Nagoya University).

(ii) pTOK232

1. Insertion of Intron GUS and Hygromycin-resistant Genes to Intermediate Vector pTOK229

The Cla I fragment (2.5 kb) of the spectinomycin-resistant gene originated from Tn7 were treated with Klenow fragment to blunt the ends. The resulting fragment was inserted in Sma I site of pUC19 to obtain a plasmid pTOK107 (5.2 kb) having ampicillin-registant and spectinomycin-resistant genes. The obtained pTOK107 was treated with Eco RI and Hind III and the obtained 2.5 kb fragment containing the spectinomysin-resistant gene was ligated to a Eco RI - Hind III fragment (2.7 kb) of pGA482 to obtain pTOK170 (5.2 kb) containing the spectinomycin-resistant gene and has Hind III site and Hpa I site.

A vector pIG221 in which the first intron of catalase of caster bean and GUS gene are ligated to 35S promoter (Ohta S. et al., 1990; Plant Cell Physiol. 31: 805-813, presented by Dr. Nakamura of Nagoya University) was digested with Eco RI and the resultant was treated with Klenow fragment to blunt the ends. To the resultant, a Hind III linker (pCAAGCTTG, code 4660P commercially available from TAKARA SHUZO). A fragment containing 35S promoter and intron GUS was cut out by digesting the resulting vector with Hind III, and the fragment was inserted into the Hind III site of a plasmid pGL2 (J. Paszkowski, obtained from Friedrich Mieocher Institute) containing a hygromycin-resistant gene ligated to 35S promoter, to obtain pGL2-IG (7.6 kb). The above-mentioned plasmid pGL2 was obtained by inserting a hygromycin-resistant gene (Gritz L. and Davis J. 1983; Gene 25: 179 - 188) into pDH51 (Pietrazak et al., 1986; Nucleic Acids Research 14: 5857 - 5868). The fragment obtained by treating pTOK170 with Hpa I was ligated to a Pvu II fragment (5.2 kb) of pGL2-IG to obtain pTOK229 (10.1 kb).

2) Insertion into Super Binary Vector pTOK162

Insertion of the desired genes (hygromycin-resistant gene and intron GUS gene) into a super binary vector pTOK162 obtained by inserting virB, virC and virG genes of strongly virulent Agrobacterium tumefaciens A281 into the binary vector was carried out by homologus recombination. That is, since both vectors contain a region originated from an E. coli plasmid pBR322, in the bacterial cells selected by resistances to spectinomycin and kanamycin, only the plasmid generated by recombination of the both plasmids are contained. The plasmid obtained by the fact that the hygromycin-resistant gene and the intron GUS gene were inserted into the super binary vector is called pTOK232 (see Fig. 1).

(3) Host Agrobacterium tumefaciens

Strains LBA4404 and EHA101 in which T-DNA regions were deleted were used as the host bacteria. Strain LBA4404 has a helper plasmid PAL4404 (having a complete vir region), and is available from American Type Culture Collection (ATCC 37349). Strain EHA101 has a helper plasmid having the vir region originated from a strongly virulent Agrobacterium tumefaciens A281, and is available from Hood E.E. et al., 1986.

The various binary vectors described in (2) were introduced into these two strains of Agrobacterium tumefaciens, and the strains described in the following were used for introducing the genes. The plasmids were introduced into the Agrobacterium strains by triple cross (Ditta G. et al., 1980; Proc. Natl. Acad. Sci.

(iii) Cultured Tissue (Scutellum Callus)

Tissues cultured with the Agrobacterium strains for 3 days were cultured on 2N6 medium containing 250 mg/l of cefotaxime for 1 week. Hygromycin-resistant cultured tissues were selected by culturing the cultured tissues on 2N6 medium containing 50 mg/l of hygromycin for 3 weeks (primary selection). The obtained resistant tissues were further cultured on N6-12 medium (N6 inorganic salts, N6 vitamins, 2 g/l of casamino acid, 0.2 mg/l of 2,4-D, 0.5 mg/l of 6BA, 5 mg/l of ABA, 30 g/l of sorbitol, 20 g/l of sucrose and 2 g/l of Gelrite) containing 50 mg/l of hygromycin for 2 - 3 weeks (secondary selection), and the calli grown on this medium were transferred to a plant regeneration medium N6S3 containing 0, 20 or 50 mg/l of hygromycin. In all of the media used after the culture with Agrobacterium strains, cefotaxime was added to 250 mg/l.

(iv) Suspended Cultured Cells

The cells cultured with the Agrobacterium strains for 5 days were cultured in 2N6 medium containing 250 mg/l of cefotaxime for 1 week, and then the selection of the transformed cells was carried out on 2N6 medium containing 50 mg/l of hygromycin.

(8) Expression of Introduced Genes in the Progeny of Transformants

Seeds of the progeny of the transformants were sown in aqueous 400-fold Homai hydrate (Kumiai Kagaku Inc.) solution containing 70 mg/l of hygromycin and incubated therein at 25°C for 10 days, thereby examining the resistance to hygromycin. Twenty seeds of each plant of the progeny of the transformants were sown and cultured for about 3 weeks. From the obtained seedlings, leaves were collected and examined for the expression of GUS gene.

(9) Analysis of Introduced Genes by Southern Blotting Method

From the primary transformants of varieties Asanohikari and Tsukinohikari, DNAs were extracted by the method of Komari et al. (Komari et al., 1989; Theoretical and Applied Genetics 77: 547-552), and the DNAs were treated with a restriction enzyme Hind III. The resulting fragment was subjected to detection of the introduced genes by Southern blotting analysis using the HPT gene as a probe. The length of the Hind III fragment containing the HPT gene as a probe is about 5.5 kb and the length of the DNA region from the Hind III site in the T-DNA in this region to the L border sequence is about 5.4 kb (Fig. 1). The Southern blotting analysis was carried out in accordance with Molecular Cloning (Sambrook et al., 1989; Cold Spring Harbor Laboratory Press). Two GUS positive plants, two GUS negative plants and two hygromycin-resistant plants were picked up from each of the two lines of the progeny of transformants of Tsukinohikari and were subjected to the Southern blotting analysis in the same manner as mentioned above.

(10) Differences in Efficiencies of Introducing Genes Depending on Sample Tissues (Expression of GUS after Culturing with Agrobacterium Strains)

In order to confirm that Agrobacterium can introduce genes into cells of monocotyledons, various tissues of the rice variety Tsukinohikari were treated with Agrobacterium tumefaciens EHA101 having a super-virulent vir region, into which the binary vector (supra) containing the hygromycin-resistant gene and the GUS gene were introduced, and then the GUS activities were examined. The sample tissues were shoot apices, radicles, scutella, radicle calli, scutellum calli and suspended cultured cells. In cases where the tissues were not treated with the Agrobacterium strain, no tissues exhibited GUS activity indicated by blue color. On the other hand, in cases where the tissues were treated with Agrobacterium tumefaciens EHA101 (pIG121Hm), in all of the tissues except for radicles, expression of GUS was confirmed. The ratio of the number of the tissues showing blue color to the number of treated tissues was the highest in scutellum calli (Table 1). Further, the size of the tissues expressing GUS was also largest in scutellum calli. The tissues exhibited the second highest rate of introduction next to the scutellum calli were shoot apices. Further, while the scutellum calli and the suspension cells which are dedifferentiated tissues of scutella exhibited high introduction rate, the introduction rate in scutella was apparently lower. This suggests that genes are more easily introduced into tissues having high cell-dividing activities.

Table 2

Differences in Rate of Energence of Transformed Tissues and Cells Depending on Sample Material (Variety:Tsukinohikari)		
Sample Tissue	Number of Hygromycin-resistant Tissues /Number of Treated Tissues (%)	
	Non-Treated Group	Treated Group
Shoot Apex	0/ 20 (0)	0/ 77 (0)
Scutellum	0/ 30 (0)	0/128 (0)
Scutellum Callus	0/ 50 (0)	169/743 (23)
Suspension Cells	0/250 (0)	22/254 (9)

(12) Differences in Efficiencies of Introducing Genes Depending on Varieties of Rice (Expression of GUS after Culturing with Agrobacterium Strains)

There are large differences among varieties about the conditions for establishing cultured cells and for regenerating plants from the cultured cells (Mikami and Kinoshita 1988; Plant Cell Tissue Organ Cult. 12:311 - 314). It is said that Koshihikari is difficult to culture among the Japonica rices. On the other hand, Tsukinohikari employed in the preceding section is relatively easy to culture. When using the transformation method utilizing Agrobacterium, it is practically inconvenient if such differences among varieties exist. In order to clarify this point, the differences in the efficiencies of gene introduction between Koshihikari and Tsukinohikari which have different easiness to culture were examined. The sample tissues employed were scutellum calli and the Agrobacterium tumefaciens strains employed were EHA101(pIG121Hm) and LBA4404(pIG121Hm).

While GUS activity was observed in not less than 90% of calli of Tsukinohikari in each experiment, the GUS activity was observed in Koshihikari at lower rates (Table 3). Thus, in cases where EHA101-(pIG121Hm) or LBA4404(pIG121Hm) is used, there is a difference in the introduction efficiency between the varieties.

Table 3

Differences in Rate of Introduction of GUS Gene Depending on Agrobacterium Strain and Rice Variety				
		Number of GUS + Tissues / Number of Treated Tissues (%)		
		Strain		
Variety	Experiment	LBA4404 (pIG121Hm)	EHA101 (pIG121Hm)	LBA4404 (pTOK232)
Tsukinohikari	1	67/70 (96)	78/87 (90)	64/66(97)
Tsukinohikari	2	72/86 (84)	68/73 (93)	82/82(100)
Koshihikari	1	46/135(34)	43/116(37)	124/131(95)
Koshihikari	2	28/107(26)	81/143(57)	102/103(99)

(13) Differences in Efficiencies of Introducing Genes Depending on Agrobacterium Strains (Expression of GUS Gene after Culturing with Agrobacterium Strains)

EHA101(pIG121Hm) has a helper plasmid containing the vir region of super-virulent Agrobacterium tumefaciens A281. LBA4404(pIG121Hm) has an ordinary vir region. On the other hand, although the vir region of the helper plasmid in LBA4404(pTOK232) is ordinary, a gene which is a part of the vir region of the super-virulent Agrobacterium tumefaciens A281 is contained in the binary vector. This binary vector is originated from pTOK162 and made it possible to transform at a very high rate dicotyledonous species which are difficult to transform (Saito Y. et al., 1992; Theor. Appl. Genet. 83:679-683). Thus, there is a possibility that the transformation efficiency is largely influenced by the existence a super-virulent vir region or by the manner of existence thereof. Thus, using the above-described three Agrobacterium strains whose

Table 5

Expression of GUS Gene in Plants Regenerated from Hygromycin-resistant Calli (Variety:Asanohikari)				
Resistant Callus	Number of Regenerated Plants	Expression of GUS Gene		
		Stably Positive	Chimera	Negative
1	26	25	1	0
2	8	7	1	0
(Hygromycin was added to culture medium until regeneration of plants.)				

Table 6

Expression of GUS Gene in Plants Regenerated from Hygromycin-resistant Calli (Variety:Tsukinohikari)			
Sample Strain	Number of Lines		
	Sample Hygromycin -resistant Calli	Calli Yielded Regenerated Plants	GUS Positive Regenerated Plants
LBA4404(pIG121Hm)	3	1	1
EHA101(pIG121Hm)	20	17	10
LBA4404(pTOK232)	20	15	12
(Hygromycin was added to culture medium until regeneration of plants.)			

Table 7

Expression of GUS Gene in Plants Regenerated from Hygromycin-resistant Calli (Variety:Asanohikari)			
Sample Strain	Number of Lines		
	Sample Hygromycin -resistant Calli	Calli Yielded Regenerated Plants	GUS Positive Regenerated Plants
LBA4404(pIG121Hm)	19	5	3
EHA101(pIG121Hm)	11	4	1
LBA4404(pTOK232)	19	11	11
(Hygromycin was added to culture medium until regeneration of plants.)			

(16) Ploidy and Fertility of Seeds of Transformants

When cultivated in a green house, the thus obtained transformants exhibited normal growth and morphology and no plants exhibited characteristics of tetraploid or malformation. As for the fertility of the seeds, although some plants exhibited partial infertility or complete infertility, most plants exhibited substantially normal fertility.

(17) Expression and Analysis of Introduced Genes in the Primary Transformants and in the Progeny

Fragments obtained by Hind III digesting the whole DNAs in the primary transformants were subjected to detection of the introduced gene by Southern blotting analysis using the HPT gene as a probe. As a result, in all of the tested plants, the existence of the introduced gene in a number of 1 to several copies was confirmed (Table 8 and Table 9). While the size of the Hind III fragment containing the HPT gene in plasmid pTOK232 is 5.5 kb, in all of the tested transformants, a band having a size larger than about 6 kb

Table 8

Number of Copies of Introduced Genes in Transformants Determined by Southern Blotting Analysis and Expression of Introduced Gene in the Progeny of Transformants (Variety:Asanohikari)

Transformants	Number of Copies of Introduced Gene	Number of Plants of the Progeny of Transformants			
		Resistance to Hygromycin		Expression of GUS	
		Resitant	Sensitive	Positive	Negative
Control	-	0	60	0	20
1 - 2	2	30	0	19	1
2 - 1	2*	64	26	13	5
3 - 2	2	59	1	19	1

* In one of the two copies of the introduced genes, the restriction fragment was short, so that the introduced gene was incomplete.

Example 2

(1) Maize Varieties

Maize varieties A188, F1 (A188 x Black Mexican Sweet), F1 (A188 x B73Ht), F1 (B73Ht x A188) and F1 P3732 were selected as the sample materials. The varieties of A188, Black Mexican Sweet and B73Ht were obtained from National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry & Fisheries, and P3732 was obtained from IWATA RAKUNOU KYODOKUMIAI.

(2) Preparation of Tissues in the Vicinity of Growth Point

Mature seeds were immersed in 70% ethanol for 1 minute and in 1% sodium hypochlorite for 5 minutes. The seeds were then washed three times with sterilized water and were placed on LS solid medium (inorganic salts and vitamins of Linsmaier and Skoog; Linsmaier, E. and Skoog, F. 1965; Physiol. Plant 18: 100 - 127, 100 mg/l of casamino acid, 700 mg/l of proline, 20 g/l of sucrose and 2.3 g/l of Gelrite). After culturing the seeds at 25°Cin the dark for 4 days, tissues with a length of about 0.1 x 0.3 mm containing the apex dividing tissues were cut out and used as samples.

(3) Preparation of Calli Originated from Immature Embryos

Immature embryos were placed on LSD1.5 solid medium (inorganic salts and vitamins of Linsmaier and Skoog, 100 mg/l of casamino acid, 700 mg/l of proline, 1.5 mg/l of 2,4-D, 20 g/l of sucrose and 2.3 g/l of Gelrite). After culturing the embryos for 3 weeks, the formed calli originated from scutella were collected and used in the subsequent experiments.

(4) Strains of Agrobacterium

Among the strains of Agrobacterium described in Example 1, LBA4404(pTOK232) and EHA101-(pIG121Hm) were used.

(5) Preparation of Suspensions of Agrobacterium Strains

Colonies of the Agrobacterium strains obtained by culturing the Agrobacterium strains on AB medium containing hygromycin (50 mg/l) and kanamycin (50 mg/l) for 3 - 10 days were collected using a platinum loop and the cells were suspended in the modified AA medium described in Example 1. The cell population was adjusted to 3 x 10^9 - 5 x 10^9 cells/ml and the resultants were used for inoculation.

(6) Conditions for Inoculation to Tissues in the Vicinity of Growth Point and for Culturing

After piercing the cut out tissues with a glass needle, the sample tissues were immersed in the above-described suspensions of the Agrobacterium strains for 3 - 10 minutes. The tissues were then transferred to modified LS solid medium (inorganic salts of Linsmaier and Skoog, vitamins of Murashige and Skoog; Murashige, T. and Skoog, F. 1962; Physiol. Plant. 15:473-497, 0.1 mg/l of kinetin, 1.0 mg/l of casamino acid and 2.3 g/l of Gelrite) and were cultured at 25°C under illumination for 2 - 3 days. Thereafter, the tissues were washed with sterilized water containing 250 mg/l of cefotaxime and then continued to be cultured on LS solid medium containing the same concentration of cefotaxime.

(7) Conditions for Inoculation to Calli and for Culturing

The calli were immersed in the above-described Agrobacterium suspensions for about 5 minutes and the resulting calli were transferred to 2N6 solid medium containing acetosyringone described in Example 1 at 25°Cin the dark for 3 days to carry out the culture with the Agrobacterium strains. The calli were washed with sterilized water containing 250 mg/l of cefotaxime and then continued to be cultured on LSD1.5 solid medium containing the same concentration of cefotaxime and 30 mg/l of hygromycin, thereby carrying out the selection of transformed calli.

Table 11

Efficiency of Introduction of GUS Gene into Maize Calli		
Variety	Strain	Number of GUS+ Calli / Number of Treated Calli (%)
A188	1	32/35 (91)
A188	1	34/34 (100)
A188xBMS	1	41/49 (84)
A188xB73	1	35/42 (83)
A188	2	39/40 (98)
A188	2	40/40 (100)
A188xBMS	2	38/40 (95)
A188xB73	2	31/40 (78)
B73 x A188	2	29/35 (83)
BMS : Black Mexican Sweet Strain 1 : EHA101(pIG121Hm), 2 : LBA4404(pTOK232)		

Claims

1. A method for transforming a monocotyledon comprising transforming a cultured tissue during dedifferentiation process or a dedifferentiated cultured tissue of said monocotyledon with a bacterium belonging to genus Agrobacterium containing a desired gene.

2. The method according to claim 1, wherein said monocotyledon is a plant belonging to family Gramineae.

3. The method according to claim 1, wherein said monocotyledon is rice.

4. The method according to claim 1, wherein said monocotyledon is corn.

5. The method according to any one of claims 1 - 4, wherein said bacterium belonging to genus Agrobacterium contains Ti plasmid or Ri plasmid, which bacterium has a plasmid containing a DNA fragment originated from the virulence region of a Ti plasmid pTiBo542 of Agrobacterium tumefaciens.

6. The method according to claim 5, wherein said plasmid containing said DNA fragment is pTOK162 or a derivative thereof.

7. The method according to any one of claims 1 - 6, wherein said bacterium belonging to genus Agrobacterium is Agrobacterium tumefaciens.

8. The method according to any one of claims 1 - 7, wherein cell population of said bacterium belonging to genus Agrobacterium used for transformation is 10^6 - 10^{11} cells/ml.

9. The method according to any one of claims 1 - 8, wherein said cultured tissue is subjected to transformation without a pretreatment in which said cultured tissue is treated with an enzyme or in which said cultured tissue is injured.

10. The method according to any one of claims 1 - 9, further comprising a step of selecting a transformed cell or a transformed tissue during dedifferentiation process or in dedifferentiated state, after subjecting said cultured tissue to transformation.

11. The method according to any one of claims 1 - 9, wherein said cultured tissue is a cultured tissue during callus formation process which is attained by culturing a explant on a differentiation medium for not less than 7 days.

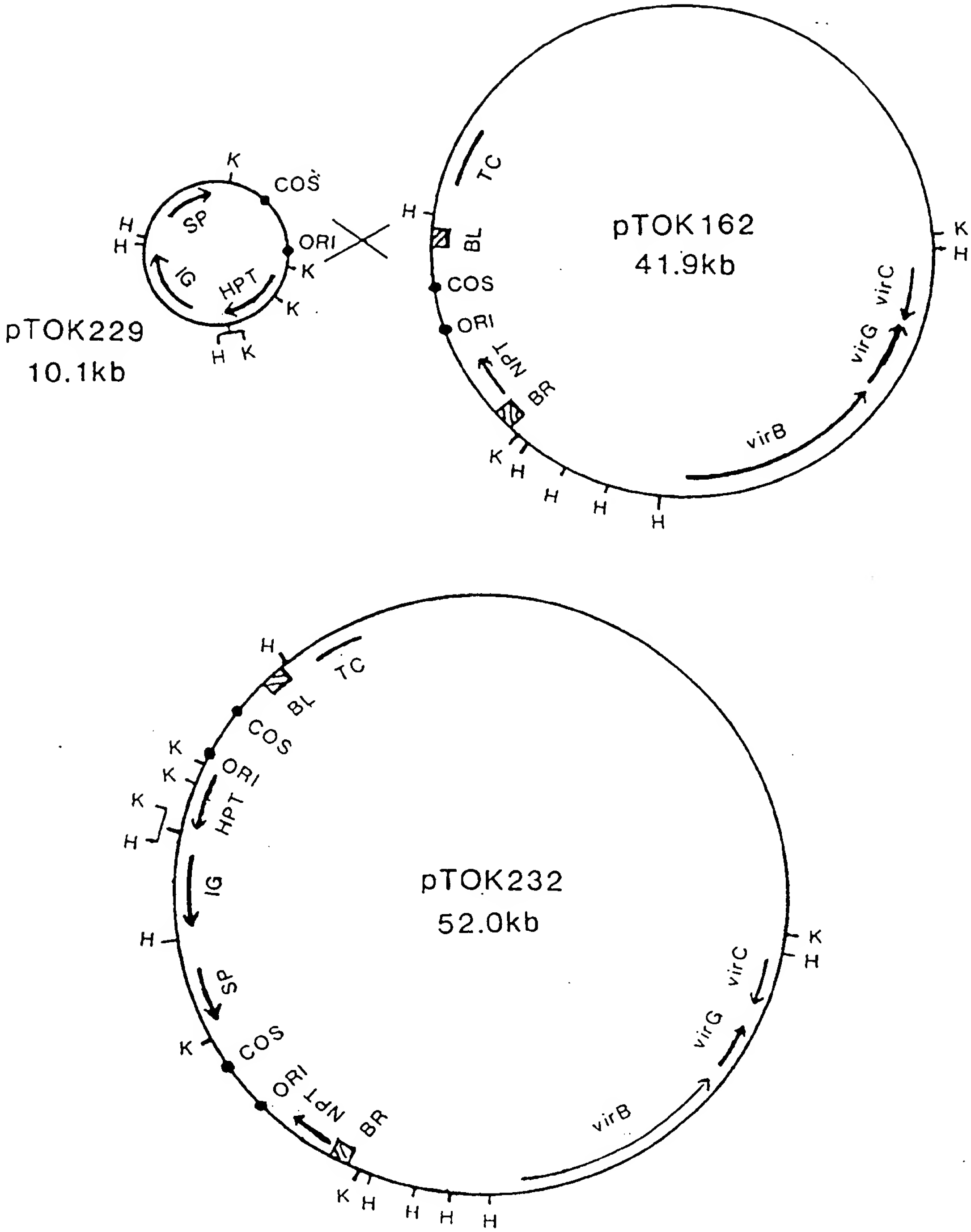
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FIG. 1